

## Research Article

# Granulocyte–macrophage colony-stimulating factor activates the transcription of nuclear factor kappa B and induces the expression of nitric oxide synthase in a skin dendritic cell line

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**Summary** Nitric oxide (NO) produced by skin dendritic cells and keratinocytes plays an important role in skin physiology, growth and remodelling. Nitric oxide is also involved in skin inflammatory processes and in modulating antigen presentation (either enhancing or suppressing it). In this study, we found that GM-CSF stimulates the expression of the inducible isoform of nitric oxide synthase (iNOS) in a fetal-skin-derived dendritic cell line (FSDC) and, consequently, increases the nitrite production from  $11.9 \pm 3.2 \mu\text{mol/L}$  (basal level) to  $26.9 \pm 4.2 \mu\text{mol/L}$ . Pyrrolidinedithiocarbamate (PDTC) inhibits nitrite production, with a half maximal inhibitory concentration ( $\text{IC}_{50}$ ) of  $19.3 \mu\text{mol/L}$  and the iNOS protein expression in FSDC. In addition, western blot assays revealed that exposure of FSDC to GM-CSF induces the phosphorylation and degradation of the inhibitor of NF- $\kappa$ B (I $\kappa$ B), with subsequent translocation of the p50, p52 and RelB subunits of the transcription nuclear factor kappa B (NF- $\kappa$ B) from the cytosol to the nucleus. Electrophoretic mobility shift assays (EMSA) showed that FSDC exposure to GM-CSF activates the transcription factor NF- $\kappa$ B. Together, these results show that GM-CSF induces iNOS expression in skin dendritic cells by a mechanism involving activation of the NF- $\kappa$ B pathway.

**Key words:** GM-CSF, NF- $\kappa$ B, nitric oxide, nitric-oxide synthase, skin dendritic cell.

## Introduction

The epidermal skin dendritic cells (DC) are antigen-presenting cells. They capture the antigen and then leave the epidermal environment and migrate to the lymph nodes, where they transfer the information to the cells of the adaptive immune response. The process of DC migration is a tightly regulated event that is dependent on epidermal cytokines, namely IL-1, TNF- $\alpha$  and GM-CSF, which induce phenotypic and functional changes in DC.<sup>1,2</sup>

Nitric oxide (NO) is a highly reactive radical produced from the aminoacid L-arginine by the enzyme NO synthase (NOS). Three NOS isoforms have been identified. Two distinct NOS isoforms are constitutively expressed in cells, whereas a third isoform, inducible NOS (iNOS), is transcribed in response to cytokines and bacterial endotoxin LPS.<sup>3</sup> Nitric oxide has been found to have a critical role in the development of immune skin reactions.<sup>4–9</sup> Expression of the iNOS isoform, promoted by LPS and IFN- $\gamma$ , has been demonstrated in the epidermal cells, keratinocytes and Langerhans cells (LC), and in bone-marrow-derived DC,<sup>5,10–12</sup> but the involvement of GM-CSF in the iNOS protein expression has not been addressed before.

The promoter region of the iNOS gene contains binding

sites for the transcription nuclear factor kappa B (NF- $\kappa$ B). Five members of the mammalian NF- $\kappa$ B/Rel family encoding the proteins p50, p52, p65 (RelA), RelB and c-Rel, have been cloned and characterized. In resting cells, NF- $\kappa$ B proteins are localized in the cytosol in association with inhibitory proteins called I $\kappa$ B.<sup>13</sup> Cell activation by various inducers results in I $\kappa$ B protein phosphorylation and degradation which, in turn, leads to NF- $\kappa$ B protein translocation to the nucleus, where they upregulate iNOS gene expression.<sup>14</sup>

We have previously reported that a fetal skin dendritic cell line (FSDC), which exhibits functional characteristics of a DC precursor, produces NO in response to LPS by a mechanism involving both Janus kinase 2 (JAK2) and NF- $\kappa$ B activation.<sup>15</sup> Therefore, the aim of this study was to determine whether GM-CSF induces iNOS protein expression, and whether the antioxidant inhibitor of NF- $\kappa$ B, pyrrolidinedithiocarbamate (PDTC),<sup>14</sup> inhibits the production of NO and iNOS expression induced by GM-CSF. Moreover, we also identified the family members of NF- $\kappa$ B proteins that are translocated from the cytosol to the nucleus after skin DC exposure to GM-CSF.

## Materials and Methods

### Materials

The rabbit antimouse iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY, USA), and the rabbit antihuman NF- $\kappa$ B, p65, was from Serotec (Oxford, UK). The rabbit anti I $\kappa$ B- $\alpha$  polyclonal antibody and the mouse anti phospho-I $\kappa$ B- $\alpha$  (Ser32/36) monoclonal antibody were from New England BioLabs

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Inc. (Beverly, MA, USA). The NF- $\kappa$ B consensus oligonucleotide, the rabbit antihuman NF- $\kappa$ B RelB, the rabbit antihuman NF- $\kappa$ B p50 and the rabbit antihuman NF- $\kappa$ B p52 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mouse monoclonal antibody against actin and the protease inhibitor cocktail were purchased from Roche (Carnaxide, Portugal).  $^{32}$ P-labelled  $\gamma$ -ATP, the ECL western blotting analysis system and the X-ray films were from Amersham Life Sciences (Buckinghamshire, UK) and the T4 polynucleotide kinase and poly(dI-dC).poly(dI-dC) were from Pharmacia Biotech (Carnaxide, Portugal). The horseradish peroxidase-conjugated swine antirabbit was from DAKO (Copenhagen, Denmark) and the horseradish peroxidase-conjugated goat antimouse was from Pierce (Rockford, IL, USA). The mouse rGM-CSF was from Endogen (Woburn, MA, USA), FCS was from Biochrom KG (Berlin, Germany) and trypsin from Gibco (Paisley, UK). All other reagents were from Sigma Chemical Co. (St Louis, MO, USA).

### Cell culture

The fetal mouse skin dendritic cell line, FSDC, was kindly supplied by Dr G Girolomoni (Laboratory of Immunology, Istituto Dermatologico dell'Immacolata, IRCCS, Rome, Italy).<sup>16</sup> The cells were cultured in endotoxin free Iscove's medium supplemented with 10% (v/v) FCS, 1% (w/v) glutamine, 3.02 g/L sodium bicarbonate, 100  $\mu$ g/mL streptomycin and 100 U/mL penicillin. For western blot and electrophoretic mobility shift assay (EMSA) analysis, FSDC were plated at  $2 \times 10^6$  cells/wells, in six-well culture dishes, for 24 h prior to treatment, whereas for nitrite measurements the cells were plated at  $0.2 \times 10^6$  cells/wells, in 48-well culture dishes.

### Nitrite measurement

The production of NO was accessed as the accumulation of nitrite ( $\text{NO}_2^-$ ) in the culture supernatants by using a colorimetric reaction with the Griess reagent.<sup>17</sup> Briefly, after stimulation for 48 h, the culture supernatants were collected and diluted with equal volumes of the Griess reagent (0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride, 1% (w/v) sulfanilamide and 5% (w/v)  $\text{H}_3\text{PO}_4$ ), for 10 min. The absorbance at 550 nm was measured after 10 min incubation in an automated plate reader (SLT, Salzburg, Austria). The nitrite concentration was determined from a sodium nitrite standard curve.

### Western blot analysis

For immunodetection of iNOS, the cells were pretreated for 2 h with culture medium in the presence or in the absence (control) of 30  $\mu$ mol/L PDTC. Next, the cells were treated with culture medium (control) or with GM-CSF (200 ng/mL) in the presence or in the absence of the inhibitor, for 24 h. After treatment, the cells were washed twice with PBS, and lysed with 200  $\mu$ L of lysis buffer (PBS containing 10 mmol/L EDTA, 1% (v/v) Triton X-100 and the protease inhibitor cocktail).

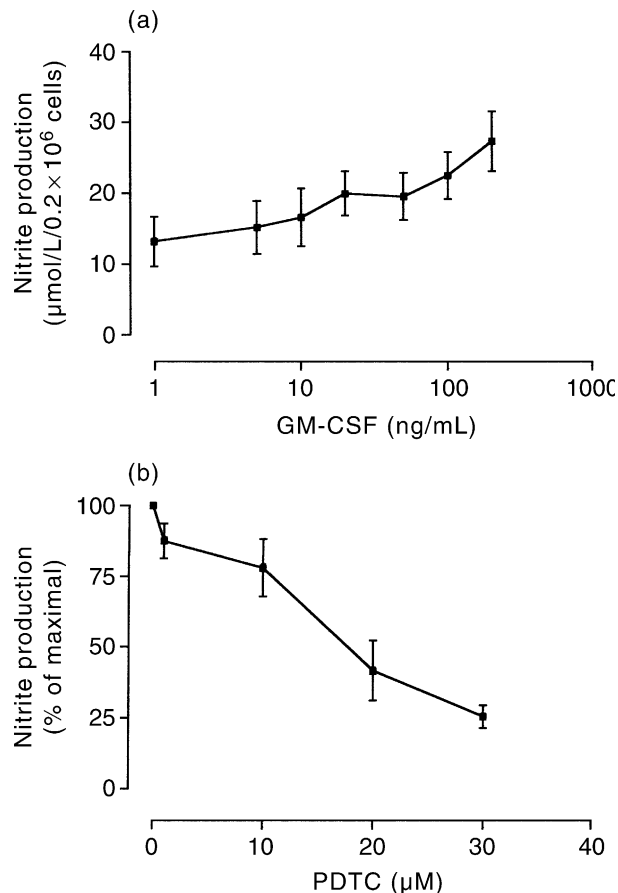
For immunodetection of I $\kappa$ B- $\alpha$ , phospho I $\kappa$ B- $\alpha$ , p50, p52, p65 and RelB, FSDC cells were treated with culture medium (control) or with GM-CSF (200 ng/mL) for 5 min, 15 min, 30 min, 1 h and 3 h. Proteins of the cytosolic fraction were obtained after harvesting the cells in 10 mmol/L NaCl, 3 mmol/L  $\text{MgCl}_2$ , 0.5% (v/v) Nonidet P-40, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonylfluoride, 10 mmol/L Tris-HCl pH 7.5, and the protease inhibitor cocktail. In addition, and for immunodetection of phospho I $\kappa$ B- $\alpha$ , 2 mmol/L of sodium orthovanadate was also used. The lysates were incubated on ice for 15 min and the cytosolic proteins were isolated from the supernatant obtained after centrifugation at 2300 g, for 10 min. In order to obtain proteins of the nuclear fraction the pellet

obtained above was resuspended in 300 mmol/L NaCl, 3 mmol/L  $\text{MgCl}_2$ , 20% (v/v) glycerol, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonylfluoride, 0.2 mmol/L EDTA, 20 mmol/L HEPES buffer (pH 7.5), and the protease inhibitor cocktail, incubated on ice for 1 h, and centrifuged at 12000 g for 20 min. The supernatant containing the nuclear proteins was collected and the protein concentration was determined using a bicinchoninic acid solution.

In brief, protein samples were separated on a 10% (v/v) (for iNOS detection) or 15% (v/v) (for I $\kappa$ B- $\alpha$ , phospho I $\kappa$ B- $\alpha$ , p50, p52, p65 and RelB detection) SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% (w/v) dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20, for 1 h. The levels of iNOS, I $\kappa$ B- $\alpha$ , phospho I $\kappa$ B- $\alpha$ , p50, p52, p65 and RelB proteins were detected using a rabbit polyclonal antimouse iNOS antibody (1:2000), a rabbit polyclonal anti-I $\kappa$ B- $\alpha$  antibody (1:1000), a mouse monoclonal anti phospho-I $\kappa$ B- $\alpha$  (Ser32/36) antibody (1:1000), a rabbit polyclonal antihuman p50 antibody (1:200), a rabbit polyclonal antihuman p52 antibody (1:500), a rabbit polyclonal antihuman p65 antibody (1:1000) and a rabbit polyclonal antihuman RelB antibody (1:500), respectively, for 1 h, followed by incubation with a horseradish peroxidase-conjugated swine anti-rabbit antibody (1:1000) or a horseradish peroxidase-conjugated goat antimouse antibody (1:25 000). The immunocomplexes were visualized by the ECL chemiluminescence method. To demonstrate equivalent protein loading the membrane was stripped and reprobed with an anti-actin antibody (1:10 000).

### Electrophoretic mobility shift assay

Fetal-skin-derived dendritic cell line cells were treated for 30 min and 1 h with GM-CSF (200 ng/mL). The cells were then washed with PBS and lysed in 10 mmol/L NaCl, 3 mmol/L  $\text{MgCl}_2$ , 0.5% (v/v) Nonidet P-40, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonylfluoride, 10 mmol/L Tris-HCl (pH 7.5), and the protease inhibitor cocktail. The lysates were incubated on ice for 15 min and centrifuged at 2300 g for 10 min. The pellet obtained was resuspended in 300 mmol/L NaCl, 3 mmol/L  $\text{MgCl}_2$ , 20% (v/v) glycerol, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonylfluoride, 0.2 mmol/L EDTA, 20 mmol/L HEPES buffer (pH 7.5), and the protease inhibitor cocktail, incubated on ice for 1 h, and centrifuged at 12000 g for 20 min. The supernatant containing the nuclear proteins was collected, and the protein concentration was determined using a bicinchoninic acid solution. The EMSA method used was similar to that described previously,<sup>14</sup> with slight modifications. The probes consisted of a double-stranded oligonucleotide containing the consensus binding sequence for NF- $\kappa$ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') end-labelled with [ $\gamma$ - $^{32}$ P]ATP using T4 polynucleotide kinase. Typical binding reactions consisted of 12  $\mu$ g of nuclear extract, ~200 000 cpm of [ $\gamma$ - $^{32}$ P]-labelled oligonucleotide, 100  $\mu$ g/mL poly(dI-dC).poly(dI-dC) in a buffer containing 20 mmol/L HEPES (pH 7.9), 1 mmol/L  $\text{MgCl}_2$ , 4% (w/v) Ficoll 400, 0.5 mmol/L dithiothreitol, 50 mmol/L KCl, and 1 mg/mL BSA, and were incubated at room temperature for 45 min. Binding reactions were separated on 10% (v/v) non-denaturing polyacrylamide gels, in a buffer system containing 0.044 mol/L Tris-Base (pH 8.0), 4.45 mmol/L boric acid and 1 mmol/L EDTA, at a constant voltage of 150 V, for 2 h at room temperature. The gels were transferred to Whatman paper, dried and subjected to autoradiography. In competition experiments, unlabelled oligonucleotide was added to the nuclear extracts for 30 min before addition of the radiolabelled probe. To detect supershifted bands, antip50, antip52, antip65 and anti-RelB antibodies (2  $\mu$ g) were incubated with the nuclear extracts for 30 min before addition of the radiolabelled probe.



**Figure 1** Dose-dependent effect of pyrrolidinedithiocarbamate (PDTC) on GM-CSF-induced nitrite production in fetal-skin-derived dendritic cell line (FSDC). (a) FSDC cells ( $0.2 \times 10^6$  cells) were incubated for 48 h with different concentrations of GM-CSF (1–200 ng/mL). (b) FSDC cells ( $0.2 \times 10^6$  cells) were incubated with GM-CSF (200 ng/mL) and the indicated concentrations of PDTC for 48 h. Results are expressed as percentage of maximal nitrite production by cells maintained in culture medium in the presence of GM-CSF and in the absence of PDTC. Nitrite levels in the culture supernatants were detected by the Griess reaction as described in experimental procedures. Each value represents the mean  $\pm$  SEM from five experiments performed in duplicate.

#### Data analysis

Results are presented as mean  $\pm$  SEM of the indicated number of experiments. Mean values were compared using one-way ANOVA and the Bonferroni's multiple comparison test. The significance level was 0.05.

#### Results

##### *PDTC inhibits the production of NO and the expression of NOS in FSDC cells stimulated with GM-CSF*

The enzymatic production of NO by FSDC was evaluated by the measurement of nitrite concentration in the culture medium. As indicated in Fig. 1a, stimulation of the cells with

GM-CSF for 48 h resulted in a dose-dependent increase in nitrite production, from  $11.9 \pm 3.2 \mu\text{mol/L}$ , when FSDC were incubated with culture medium alone, to  $27.0 \pm 4.2 \mu\text{mol/L}$ , when FSDC were incubated with 200 ng/mL GM-CSF.

The contribution of NF- $\kappa$ B in GM-CSF-induced NO production was examined by measuring the effect of PDTC, an antioxidant inhibitor of the transcription factor NF- $\kappa$ B,<sup>14</sup> on nitrite production after stimulation of the cells with GM-CSF (Fig. 1b). The results indicate that PDTC elicited concentration-dependent inhibition of GM-CSF-induced nitrite formation in FSDC cells, with an  $\text{IC}_{50}$  value of  $19.3 \mu\text{mol/L}$ , as calculated by the Hill plots (Fig. 1b). The assay of cellular 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction did not show any significant toxic effect induced by PDTC for the concentrations used in the experiments above (data not shown).

Western blot was used to examine whether GM-CSF induces the expression of iNOS protein (130 kDa). As indicated in Fig. 2 (lane 1), non-stimulated cells expressed low levels of iNOS protein, but the expression of the protein increased when the cells were stimulated with 200 ng/mL GM-CSF for 24 h (Fig. 2; lane 2). PDTC (30  $\mu\text{mol/L}$ ) inhibited the expression of iNOS in cells stimulated with GM-CSF (Fig. 2; lane 3). This decrease in protein expression caused by PDTC correlated well with the maximal inhibitory effect on NO production, as shown in Fig. 1b.

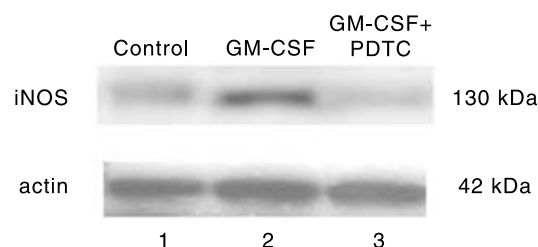
##### *GM-CSF induces the phosphorylation and degradation of cytosolic I $\kappa$ B- $\alpha$ and subsequent translocation of NF- $\kappa$ B p50, p52 and RelB proteins from the cytosol to the nucleus in FSDC cells*

To determine whether GM-CSF induced I $\kappa$ B- $\alpha$  phosphorylation and degradation, the levels of phospho-I $\kappa$ B- $\alpha$  (ser32/36) and I $\kappa$ B- $\alpha$  on the cytosolic extracts were examined by western blot. As shown in Fig. 3a, treatment of cells with 200 ng/mL GM-CSF (lane 2–4), for 5 min and 15 min, caused the phosphorylation of I $\kappa$ B- $\alpha$  and consequent degradation of this protein. The effect of GM-CSF was transient because when the cells were incubated with the cytokine for 1 h, newly synthesized I $\kappa$ B- $\alpha$  accumulated in the cytosol (Fig. 3a; lane 5). The basal phosphorylation of I $\kappa$ B- $\alpha$  in control cells (lane 1) is probably due to culture FSDC cells with medium containing 10% FCS, which activates the cells per se.

To evaluate the involvement of the NF- $\kappa$ B family members in the response of FSDC to GM-CSF, the cytosolic and nuclear extracts were subjected to western blot analysis, by using antibodies against the proteins of the NF- $\kappa$ B family, p50, p52, p65 and RelB. As shown in Fig. 3b–d, 30 min and 1 h of cell stimulation with GM-CSF (200 ng/mL) resulted in a significant reduction in the cytosolic level and an increase in the nuclear level of NF- $\kappa$ B proteins p50, p52 and RelB. Treatment of FSDC with GM-CSF (200 ng/mL) did not significantly modify the level of p65 protein in the cytosol or in the nucleus (data not shown).

##### *GM-CSF induces NF- $\kappa$ B binding to DNA in FSDC cells*

Stimulation of FSDC with GM-CSF (200 ng/mL) induced NF- $\kappa$ B binding to DNA (Fig. 4), within a period of 30 min to



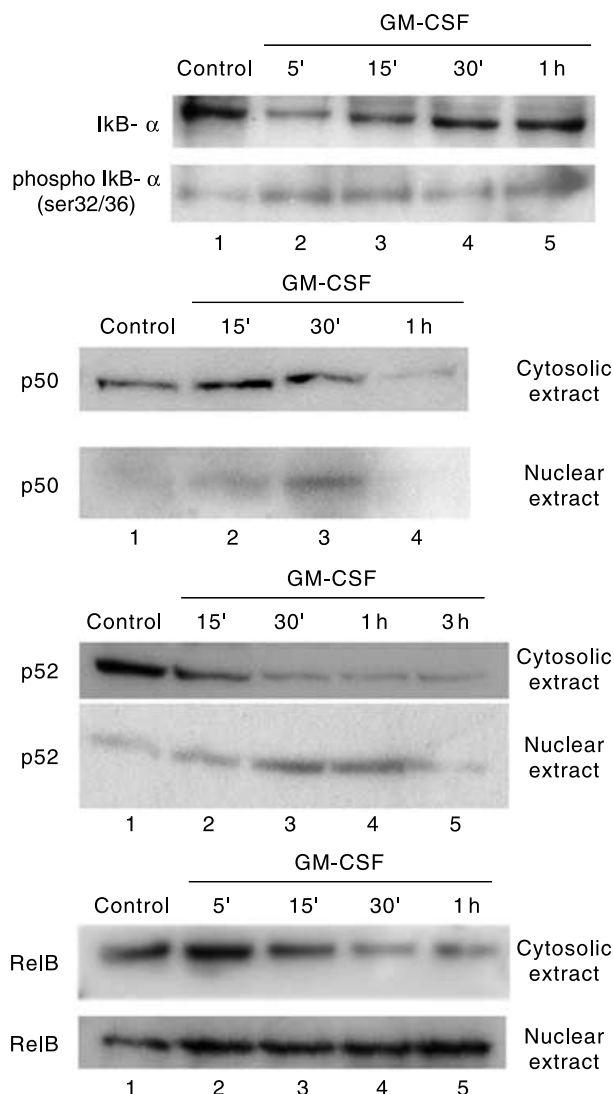
**Figure 2** Effect of pyrrolidinedithiocarbamate (PDTC) on GM-CSF-induced isoform of nitric oxide synthase (iNOS) protein expression in fetal-skin-derived dendritic cell line (FSDC). FSDC cells ( $2 \times 10^6$  cells) were incubated in culture medium alone (control, lane 1), or in the presence of 200 ng/mL GM-CSF for 24 h (lanes 2 and 3). Stimulation with GM-CSF was carried out in the absence (lane 2) or in the presence of 30  $\mu$ mol/L PDTC (lane 3). When the effect of PDTC was tested, the cells were pre-incubated with the inhibitor for 2 h before stimulation with GM-CSF. Total cell extracts were electrophoresed through SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes and subjected to western blot analysis using an anti-iNOS, as described in the experimental procedures. The membrane was stripped and reprobed with an anti-actin antibody to confirm equal protein loading. The blot shown is representative of three blots yielding similar results. The blot was digitally generated using an HP ScanJet 5p and processed in the Corel Photo-Paint program.

1 h (lane 6–7). Supershifts experiments using antibodies against the subunits of NF- $\kappa$ B were performed, and indicated that the antibodies against p50 (lane 3), p52 (lane 4) and Rel B (lane 5) proteins decrease NF- $\kappa$ B complex formation. These results are in agreement with those obtained with the western blot assay, which demonstrated that GM-CSF induced translocation of the NF- $\kappa$ B p50, p52 and RelB proteins from the cytosol to the nucleus in FSDC cells. As a control for the gel shift assays, unlabelled oligonucleotide (100-fold in excess) was used (lane 8), which inhibited NF- $\kappa$ B complex formation.

## Discussion

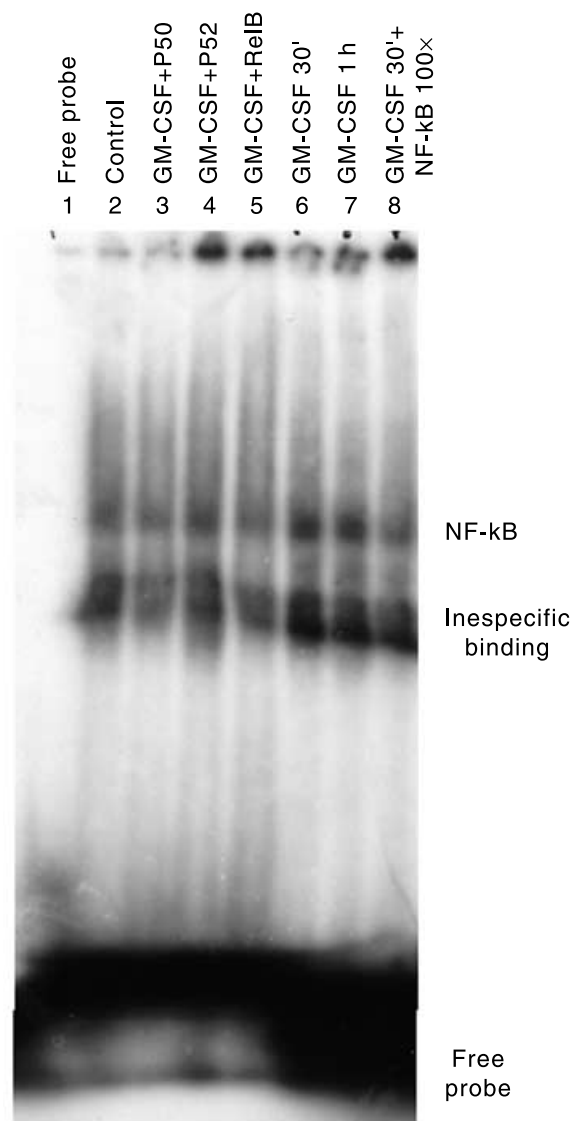
In the present study, we show that GM-CSF induces NO production and iNOS expression in FSDC (Figs 1a,2). Our results indicate that the antioxidant inhibitor of NF- $\kappa$ B, PDTC, inhibited, in a dose-dependent fashion, iNOS expression and nitrite production induced by GM-CSF in FSDC (Figs 1b,2). Moreover, exposure of FSDC to GM-CSF induced phosphorylation and degradation of I $\kappa$ B- $\alpha$  with subsequent translocation of the NF- $\kappa$ B proteins (p50, p52 and RelB) into the nucleus (Fig. 3) to bind DNA (Fig. 4).

The list of immunological agents known to induce iNOS gene expression *in vivo* and *in vitro* has grown in the past few years,<sup>3</sup> however, some disagreement still exists concerning the effect of GM-CSF in iNOS expression. For example, in alveolar macrophages, GM-CSF increased nitrite formation,<sup>18</sup> whereas in cartilage chondrocytes and in peritoneal macrophages GM-CSF had no effect on nitrite production.<sup>19,20</sup>



**Figure 3** Granulocyte-macrophage colony-stimulating factor induced cytosolic phosphorylation and degradation of the inhibitor of NF- $\kappa$ B (I $\kappa$ B- $\alpha$ ) and proteins nuclear factor kappa B (NF- $\kappa$ B), p50, p52 and RelB translocation from the cytosol into the nucleus in fetal-skin-derived dendritic cell line (FSDC) cells. FSDC cells ( $2 \times 10^6$  cells) were incubated with culture medium alone (control, lanes 1) or with GM-CSF (200 ng/mL) for the time periods indicated in the figure. Cytosolic and nuclear cell extracts were electrophoresed through SDS-PAGE and subjected to western blot analysis using anti-I $\kappa$ B- $\alpha$  and (a) antiphospho-I $\kappa$ B- $\alpha$ , (b) antip50, (c) antip52 and (d) anti-RelB antibodies, as described in the experimental procedures. The blots shown are representative of three blots yielding similar results. The blots were digitally generated using an HP ScanJet 5p and processed in the Corel Photo-Paint program.

Stimulation of iNOS expression by LPS and IFN- $\gamma$  was shown in both cell types, bone marrow-derived-DC<sup>10</sup> and skin LC,<sup>11,12</sup> in contrast to the lack of effect of IFN- $\gamma$  + LPS on the expression of iNOS mRNA in mouse epidermal LC.<sup>21</sup> The intracellular signalling pathways by which GM-CSF induces



**Figure 4** Granulocyte-macrophage colony-stimulating factor induced nuclear factor kappa B (NF- $\kappa$ B) activation in fetal-skin-derived dendritic cell line (FSDC) cells. FSDC cells ( $2 \times 10^6$  cells) were incubated, for the time periods indicated, in culture medium alone (control, lane 2), or in the presence of 200 ng/mL GM-CSF, for 30 min and 1 h (lane 6–7). Nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) analysis as described in the experimental procedures. Supershift experiments were done by using specific antip50, antip52 and anti-RelB antibodies (lanes 3–5). To demonstrate specificity of induced bands, binding was carried out in the presence of a molar excess (100 $\times$ ) of non-radioactive NF- $\kappa$ B consensus containing oligonucleotide (lane 8). The gel shown is representative of three gels yielding similar results. The gel was digitally generated using an HP ScanJet 5p and processed in the Corel Photo-Paint program.

iNOS expression in DC are not known. Because the promoter region of the iNOS gene contains binding sites for NF- $\kappa$ B,<sup>22</sup> it is possible that in FSDC GM-CSF induces iNOS expression through NF- $\kappa$ B activation. Accordingly, our results show

that PDTC prevented GM-CSF-induced iNOS expression (Fig. 2) and nitrite production by FSDC (Fig. 1b), indicating that NF- $\kappa$ B participates in GM-CSF-induced iNOS expression. Moreover, we demonstrated that GM-CSF translocates the NF- $\kappa$ B proteins p50, p52 and RelB into the nuclei of skin DC. Activation of the transcription factor NF- $\kappa$ B by GM-CSF was also reported during the erythropoiesis in human erythroid precursors.<sup>23</sup> We have previously reported that in FSDC, LPS induces I $\kappa$ B- $\alpha$  degradation and translocates the NF- $\kappa$ B p65 protein into the nucleus,<sup>15</sup> which indicates that in skin DC the activation of NF- $\kappa$ B represents a crucial step in the induction of iNOS, as previously reported in other cell types.<sup>22</sup> In contrast, in this study, GM-CSF activates p50, p52 and Rel B and has no significant effect on the p65 subunit of NF- $\kappa$ B, probably because the signalling pathways activated by LPS or GM-CSF, which in turn activate the NF- $\kappa$ B proteins, are different. In FSDC cells, the amount of NO production induced by GM-CSF is four-fold lower than that produced by the cells incubated with LPS.<sup>12</sup>

The GM-CSF-induced signalling pathway(s) responsible for NF- $\kappa$ B activation and iNOS expression in DC, has not yet been identified. In neutrophils and haematopoietic progenitors, GM-CSF activates different signalling pathways, namely mitogen activated protein kinases, the JAK/STAT and the phosphatidylinositol 3-kinase.<sup>24–27</sup> A direct link from phosphatidylinositol 3-kinase to NF- $\kappa$ B activation via an I $\kappa$ B kinase, which phosphorylates I $\kappa$ B and leads to its degradation, was recently demonstrated.<sup>28</sup> These results suggest that in FSDC, GM-CSF may activate protein kinases which, in turn, activate the I $\kappa$ B kinase complex, leading to NF- $\kappa$ B activation and iNOS expression.

In the present study, we found that GM-CSF selectively induces the translocation of the p50, p52 and RelB protein subunits of NF- $\kappa$ B from the cytosol to the nucleus in FSDC (Fig. 3b–d). In normal epidermis, the NF- $\kappa$ B proteins p50 and p52, in addition to p65, are generally expressed in the cytoplasm of basal cells,<sup>29,30</sup> and NF- $\kappa$ B proteins are involved in DC differentiation and maturation.<sup>31–34</sup> The NF- $\kappa$ B member, RelB, is also involved in DC differentiation<sup>35,36</sup> and in the transactivation of genes of central importance for functional antigen-presenting cells.<sup>37–39</sup> GM-CSF was found to upregulate skin DC expression of the costimulatory molecules CD80 and CD86, which are involved in the process of T-cell activation.<sup>40</sup> These results are consistent with our findings showing that GM-CSF activates the NF- $\kappa$ B proteins, namely RelB, in these cells (Fig. 3).

Significant physiological and physiopathological consequences are believed to be the result of NO production by skin cells. For example, NO appears to be involved in skin growth and remodelling,<sup>41</sup> in skin inflammatory processes,<sup>7–9</sup> and in modulating antigen presentation.<sup>10,42,43</sup> Therefore, a better knowledge of the signalling pathways involved in NO production by skin DC will contribute to our understanding about cutaneous biology.

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## References

- 1 Bancherau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245–52.
- 2 Caux C. Pathways of development of human dendritic cells. *Eur. J. Dermatol.* 1998; **8**: 375–84.
- 3 Lamas S, Pérez-Sala D, Moncada S. Nitric oxide: from discovery to the clinic. *Tips* 1998; **19**: 436–8.
- 4 Krischel V, Bruch-Gerharz D, Suschek C, Kröncke K-D, Ruzicka T, Kolb-Bachofen V. Biphasic effect of exogenous nitric oxide on proliferation and differentiation in skin derived keratinocytes but not fibroblasts. *J. Invest. Dermatol.* 1998; **111**: 286–91.
- 5 Heck DE, Laskin DL, Gardner CR, Laskin JD. Epidermal growth factor suppresses nitric oxide and hydrogen peroxide production by keratinocytes. Potential role for nitric oxide in the regulation of wound healing. *J. Biol. Chem.* 1992; **267**: 21 277–80.
- 6 Thornton FJ, Schäffer MR, Witte MB *et al.* Enhanced collagen accumulation following direct transfection of the inducible nitric oxide synthase gene in cutaneous wounds. *Biochem. Biophys. Res. Commun.* 1998; **246**: 654–9.
- 7 Morhenn VB. Langerhans cells may trigger the psoriatic disease via production of nitric oxide. *Immunol. Today* 1997; **18**: 433–5.
- 8 Ross R, Gillitzer C, Kleinz R *et al.* Involvement of NO in contact hypersensitivity. *Int. Immunol.* 1997; **10**: 61–9.
- 9 Rowe A, Farrel AM, Bunker CB. Constitutive endothelial and inducible nitric oxide synthase in inflammatory dermatoses. *Br. J. Dermatol.* 1997; **136**: 18–23.
- 10 Lu L, Bonham CA, Chambers FG *et al.* Induction of nitric oxide synthase in mouse dendritic cells by IFN- $\gamma$ , endotoxin, and interaction with allogeneic T cells. Nitric oxide production is associated with dendritic cell apoptosis. *J. Immunol.* 1996; **157**: 3577–86.
- 11 Qureshi AA, Hosoi J, Xu S, Takashima A, Granstein RD, Lerner EA. Langerhans cells express inducible nitric oxide synthase and produce nitric oxide. *J. Invest. Dermatol.* 1996; **107**: 815–21.
- 12 Cruz MT, Duarte CB, Gonçalo M, Carvalho AP, Lopes MC. Involvement of JAK2 and MAPK on type II nitric oxide synthase expression in skin-derived dendritic cells. *Am. J. Physiol.* 1999; **277**: C1050–7.
- 13 Chen FE, Ghosh G. Regulation of DNA binding by Rel/NF- $\kappa$ B transcription factors: structural views. *Oncogene* 1999; **18**: 6845–52.
- 14 Heitmeier MR, Scarim AL, Corbett JA. Double-stranded RNA-induced inducible nitric-oxide synthase expression and interleukin-1 release by murine macrophages requires NF- $\kappa$ B activation. *J. Biol. Chem.* 1998; **273**: 15 301–7.
- 15 Cruz MT, Duarte CB, Gonçalo M, Carvalho AP, Lopes MC. LPS induction of I $\kappa$ B- $\alpha$  degradation and iNOS expression in a skin dendritic cell line is prevented by the Janus kinase 2 inhibitor, tyrphostin B42. *Nitric Oxide* 2001; **5**: 53–61.
- 16 Girolomoni G, Lutz MB, Pastore S, Abmann CU, Cavani A, Ricciardi-Castagnoli P. Establishment of a cell line with features of early dendritic cell precursors from fetal mouse skin. *Eur. J. Immunol.* 1995; **25**: 2163–9.
- 17 Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [ $^{15}$ N]nitrate in biological fluids. *Anal. Biochem.* 1982; **126**: 131–8.
- 18 Liu HW, Anand A, Bloch K, Christiani D, Kradin R. Expression of inducible nitric oxide synthase by macrophages in rat lung. *Am. J. Respir. Crit. Care Med.* 1997; **156**: 223–8.
- 19 DiNapoli MR, Calderon CL, Lopez DM. Phosphatidylserine is involved in the reduced rate of transcription of the inducible nitric oxide synthase gene in macrophages from tumor-bearing mice. *J. Immunol.* 1997; **158**: 1810–17.
- 20 Stephan S, Purcell WM, Chander CL. Colony stimulating factors regulate nitric oxide and prostaglandin E2 production in rat cartilage chondrocytes. *Int. J. Tissue React.* 1999; **21**: 113–19.
- 21 Blank C, Bogdan C, Bauer C, Erb K, Moll H. Murine epidermal Langerhans cells do not express inducible nitric oxide synthase. *Eur. J. Immunol.* 1996; **26**: 792–6.
- 22 Xie Q, Kashiwabara Y, Nathan C. Role of transcription factor NF- $\kappa$ B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* 1994; **269**: 4705–8.
- 23 Zhang M-Y, Harhaj EW, Bell L, Sun S-C, Miller BA. Bcl-3 expression and nuclear translocation are induced by granulocyte-macrophage colony-stimulating factor and erythropoietin in proliferating human erythroid precursors. *Blood* 1998; **92**: 1225–34.
- 24 Watanabe S, Itoh T, Arai K. Roles of JAK kinases in human GM-CSF receptor signal transduction. *J. Allergy Clin. Immunol.* 1996; **98**: S183–91.
- 25 Liu R, Itoh T, Arai K, Watanabe S. Activation of c-Jun N-terminal kinase by human granulocyte macrophage-colony stimulating factor in BA/F3 cells. *Biochem. Biophys. Res. Commun.* 1997; **234**: 611–5.
- 26 McDonald PP, Bovolenta C, Cassatella MA. Activation of distinct transcription factors in neutrophils by bacterial LPS, interferon- $\gamma$ , and GM-CSF and the necessity to overcome the action of endogenous proteases. *Biochemistry* 1998; **37**: 13 165–73.
- 27 Al-Shami A, Naccache PH. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Involvement of Jak2 in the stimulation of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 1999; **274**: 5333–8.
- 28 Ernfors P. Nuclear factor- $\kappa$ B to the rescue of cytokine-induced neuronal survival. *J. Cell Biol.* 2000; **148**: 223–5.
- 29 Budunova IV, Perez P, Vaden VR, Spiegelman VS, Slaga TJ, Jorcano JL. Increased expression of p50-NF- $\kappa$ B and constitutive activation of NF- $\kappa$ B transcription factors during mouse skin carcinogenesis. *Oncogene* 1999; **8**: 7423–31.
- 30 Kaufman CK, Fuchs E. It's got you covered: NF- $\kappa$ B in the epidermis. *J. Cell Biol.* 2000; **149**: 999–1004.
- 31 Ammon C, Mondal K, Andreesen R., Krause SW. Differential expression of the transcription factor NF- $\kappa$ B during human mononuclear phagocyte differentiation to macrophages and dendritic cells. *Biochem. Biophys. Res. Commun.* 2000; **268**: 99–105.
- 32 Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. *J. Exp. Med.* 1998; **188**: 2175–80.
- 33 Neumann M, Fries H, Scheicher C *et al.* Differential expression of Rel/NF- $\kappa$ B and octamer factors is a hallmark of the generation and maturation of dendritic cells. *Blood* 2000; **95**: 277–85.
- 34 Oyama T, Ran S, Ishida T *et al.* Vascular endothelial growth factor affects dendritic cell maturation through inhibition of nuclear factor- $\kappa$ B activation in hemopoietic progenitor cells. *J. Immunol.* 1998; **160**: 1224–32.
- 35 Clark GJ, Gunningham S, Troy A, Vuckovic S, Hart DN. Expression of the RelB transcription factor correlates with the activation of human dendritic cells. *Immunol.* 1999; **98**: 189–96.
- 36 St Louis DC, Woodcock JB, Fransozo G *et al.* Evidence for distinct intracellular signaling pathways in CD34+ progenitor to dendritic cell differentiation from a human cell line model. *J. Immunol.* 1999; **162**: 3237–48.

- 37 Pettit AR, Quinn C, MacDonald KPA *et al.* Nuclear localization of RelB is associated with effective antigen-presenting cell function. *J. Immunol.* 1997; **159**: 3681–91.
- 38 Burkly L, Hession C, Ogata L *et al.* Expression of *relB* is required for the development of thymic medulla and dendritic cells. *Nature* 1995; **373**: 531–6.
- 39 Lee JI, Ganster RW, Geller DA, Burckart GJ, Thomson AW, Lu I. Cyclosporine A inhibits the expression of costimulatory molecules on in vitro-generated dendritic cells: association with reduced nuclear translocation of nuclear factor kappa B. *Transplantation* 1999; **68**: 1255–63.
- 40 Yokozeki H, Takayma K, Ohki O *et al.* Comparative analysis of CD80 and CD86 on human Langerhans cells: expression and function. *Arch. Dermatol. Res.* 1998; **290**: 547–52.
- 41 Bruch-Gerharz D, Ruzicka T, Kolb-Bachofen V. Nitric oxide in human skin. current status and future prospects. *J. Invest. Dermatol.* 1998; **110**: 1–7.
- 42 Genaro AM, Hortelano S, Alvarez A, Martínez A-C, Bosca L. Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. *J. Clin. Invest.* 1995; **95**: 1884–90.
- 43 Paolucci C, Rovere P, De Nadai C, Manfredi AA, Clementi E. Nitric oxide inhibits the tumor necrosis factor alpha -regulated endocytosis of human dendritic cells in a cyclic GMP-dependent way. *J. Biol. Chem.* 2000; **275**: 19 638–44.